Ex Vivo Priming of Endothelial Progenitor Cells With SDF-1 Before Transplantation Could Increase Their Proangiogenic Potential

Faouzia Zemani, Jean-Sébastien Silvestre, Françoise Fauvel-Lafeve, Arlette Bruel, José Vilar, Ivan Bieche, Ingrid Laurendeau, Isabelle Galy-Fauroux, Anne Marie Fischer, Catherine Boisson-Vidal

Objectives—As SDF-1 and its cognate receptor CXCR4 play a key role in the survival and mobilization of immature cells, we examined whether preconditioning of endothelial progenitor cells (EPCs) with SDF-1 could further promote their capacity to enhance angiogenesis.

Methods and Results—EPC exposure to 100 ng/mL SDF-1 for 30 min induced a proangiogenic phenotype, with cell migration and differentiation into vascular cords in Matrigel and increased their therapeutic potential in a nude mouse model of hindlimb ischemia. This pretreatment enhanced EPC adhesion to activated endothelium in physiological conditions of blood flow by stimulating integrin-mediated EPCs binding to endothelial cells. Pretreated EPCs showed significantly upregulated surface α4 and αM integrin subunit expression involved in the homing of immature cells to a neovasculature and enhanced FGF-2 and promatrix metalloproteinase (MMP)-2 secretion. All these effects were significantly attenuated by EPC incubation with AMD-3100, a CXCR4 antagonist, by prior HSPGs disruption and by HUVEC incubation with anti–intercellular adhesion molecule1 (ICAM-1) and anti–vascular cell adhesion molecule (VCAM) blocking antibodies. Pretreated EPCs adhered very rapidly (within minutes) and were resistant to shear stresses of up to 2500s⁻¹.

Conclusions—SDF-1 pretreatment during EPC expansion stimulates EPC adhesion to endothelial cells and thus augments the efficiency of cell therapy for ischemic vascular diseases. (Arterioscler Thromb Vasc Biol. 2008;28:644-650)

Key Words: endothelial progenitor cells ■ SDF-1 ■ ischemia ■ HSPGs ■ adhesion

The discovery of bone marrow–derived endothelial progenitor cells (EPCs) in peripheral blood prompted an intensive search for new ways of inducing neovascularization in patients with heart and limb ischemia, based on transplantation of bone marrow– or peripheral blood–derived EPCs. Several studies indicated that local implantation of ex vivo–expanded EPCs improved neovascularization of damaged tissues in animal models of hindlimb ischemia. Contrary to differentiated endothelial cells, administration of EPCs led to increased blood flow in ischemic limbs of nude mice. These immature cells promote neovascularization by differentiating in situ into endothelial cells and by secreting growth factors, cytokines, and proteases that support angiogenic and vasculogenic processes. However, transplantation of autologous EPCs has several limitations, including the limited supply of expanded progenitors, the time required to harvest, expand, and reinject autologous EPCs, and poor graft efficiency. In addition, EPCs appear to undergo unfavorable functional changes during the expansion procedure. This may explain why the capacity of injected EPCs to promote neovascularization is highly variable and depends on the experimental model. The yield is currently so low that very large blood volumes would have to be processed to obtain sufficient EPCs for therapeutic use. The required number of EPCs can be reduced by using local infusion or by concomitant infusion of proangiogenic proteins. In addition, EPCs can be activated before their injection or mobilized into the circulation by cytokines. Cytokines released by damaged tissues mobilize bone marrow EPCs, which then migrate and promote local neovascularization. It is thought that EPCs interact with specific molecules expressed on endothelial cells then roll along the vessel wall until they come into contact with chemokines trapped in the endothelial glyocalyx. Part of this trafficking is regulated by stromal cell-derived factor 1 (SDF-1). Recent studies indicate that the interplay between this cytokine, which is present on the endothelium, and EPC adhesion molecules is the main driving force behind the...
recruitment process in vivo. SDF-1 is believed to activate EPC integrins involved in the extravasation process. SDF-1 can also induce angiogenesis in vivo and is involved in neovascularization of ischemic tissue in experimental animals. Local SDF-1 administration induces the accumulation of transplanted EPCs at sites of ischemia and enhances neovascularization after EPC transplantation.

The aim of this study was to take advantage of the proangiogenic properties of SDF-1 with a view to enhancing EPC graft efficiency. We postulated that short EPC exposure to exogenous SDF-1 might increase their transplantation efficiency by enhancing their capacity to adhere to and cross the activated endothelium, and then to differentiate.

**Methods**

An expanded methods section is available online at http://atvb.ahajournals.org.

**Cell Culture and Stimulation**

Endothelial cells from human umbilical cords (HUVECs) and EPCs from human umbilical cord blood were isolated, expanded, and characterized as previously described. One day before all experiments, cells were growth-arrested for 24 hours in EBM-2, 2% FCS, released from growth arrest by adding EBM-2, 5% FCS, with various SDF-1 concentrations for various incubation times, then washed and used in angiogenesis and adhesion assays. When needed, blocking anti-CXCR4 mAb (clone 12G5, R&D systems) or AMD3100 (10 μmol/L, Sigma) was added 30 min before the agonist SDF-1. Supernatants and cells were separately analyzed after SDF-1 stimulation. All assays were performed in triplicate.

Flow cytometry was used to assess cell surface antigen expression. Details of immunofluorescence staining are given in the Supplemental Data section (http://atvb.ahajournals.org).

Gelatin zymography of culture medium conditioned by SDF-1–treated and untreated EPCs was performed as described elsewhere, using HT1080 cell conditioned medium was used as standard. This established cell line constitutively releases pro–MMP-2 (72 kDa) and pro–MMP-9 (92 kDa) into the culture medium. Analysis was performed on culture supernatants by semiautomated image analysis expressed as gray level x surface of lysis band for 1000 cells and referred to gelatinolytic activities of HT1080 media.

**In Vitro Angiogenesis Assays**

To investigate the effect of SDF-1 on EPC proliferation, adhesion, migration, and differentiation, EPCs were stimulated as described above then detached with versene/0.01% collagenase (1/1) and washed twice with buffered Hank’s, 0.5% BSA before use in vitro angiogenesis assays. Cell outgrowth, proliferation, adhesion, migration, and in vitro tube formation were measured as previously described. For in vitro tube formation assay, pretreated EPCs were seeded on Matrigel in growth factor–depleted basal medium. After 18 h of culture the cells were fixed with glutaraldehyde and stained with Giemsa (original x20). Data were expressed as the comparison of the mean (±SEM) total length of tubules (% of control EPCs) formed in each assay.

**Real-Time Polymerase Chain Reaction**

The theoretical and practical aspects of real-time quantitative RT-PCR on the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems) have been described in detail elsewhere. The nucleotide sequences of the primers for TBP and the 10 target genes are given in the Supplemental Data section.

**Shear-Flow Adhesion Assays**

Tethering, rolling, and flow adhesion experiments were conducted with a parallel-plate flow chamber in physiological shear stress conditions as previously described. To distinguish between the adhesion of pretreated EPCs and that of detached endothelial cells, EPCs were stained with calcine (Fluoroprobes). HUVEC monolayers (7.5 x 10⁵) were seeded onto coverslips, maintained at 37°C for 6 days, placed in the flow chamber, and then stimulated by exposure to a shear rate of 50s⁻¹ for 30 min. Calcine-labeled EPCs in adhesion buffer (cation-free HBSS, 10 mmol/L HEPES, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 2 mg/mL BSA, pH 7.4) were then perfused for 15 min at 37°C at a shear rate of 50s⁻¹, and coverslips were washed with adhesion buffer for 10 min. Adherent cells were visualized by phase-contrast microscopy. All experiments were observed in real-time and videotaped for offline analysis. Images from 40 random microscope fields (1 cm²) were collected. Data were expressed as the number of adherent cells per cm². Results were pooled for 4 different experiments in each study.

The pattern of adhesion at 50s⁻¹ was also analyzed to determine the number of rolling cells (rolling across the entire field), tethering cells, and adherent cells (immediate full arrest). In this condition, the number of cells remaining adherent was expressed relative to the number of cells adhering at a shear stress of 50s⁻¹. Adherent EPCs were subjected to detachment by increasing the flow rate from 50s⁻¹ to 2500s⁻¹.

For studies requiring exogenous chemokines, HUVEC monolayers were stimulated for 30 min in flow conditions and exogenous SDF-1 (R&D systems) was added for 15 min immediately before the assay. For antibody inhibition studies, SDF-1–stimulated EPCs were perfused for 30 min on HUVECs preincubated for 30 min in adhesion buffer with the relevant mAb, anti–intercellular adhesion molecule-1 (ICAM-1) and anti–vascular cell adhesion molecule (VCAM) (Abcys). Before some flow-based adhesion assays, SDF-1–stimulated or untreated EPCs or HUVECs were pretreated for 2 h at 37°C with 0.5U/mL heparinase I (heparin lyase; Sigma), 0.1U/mL heparinase II (HS lyase; Sigma), and 0.2U/mL chondroitinases ABC (chondroitinases ABC lyase; EC4.2.2.4; Sigma).

**Mouse Hindlimb Ischemia**

Experiments were conducted according to French veterinary guidelines. All experiments used 7-week-old athymic nude mice. The mice underwent surgery to induce unilateral hindlimb ischemia as previously described and were then randomly allocated to PBS or to intravenous injection of EPCs that had or had not been treated with SDF-1. After 2 wk, the ischemic/normal limb blood flow ratio was determined by using a laser Doppler perfusion imaging system (Moor instruments). Vessel density was evaluated by high-definition microangiography using barium sulfate (1 g/mL) injected in the abdominal aorta. Capillary density in the gastrocnemius muscle was assessed by immunostaining as described elsewhere, using a rabbit antimesovine-fibronectin antibody (abcsy).

**Statistical Analysis**

Data are expressed as means±SEM of at least 3 independent experiments. Significant differences were identified by ANOVA followed by Fisher protected least significant difference test. Statistical analysis was performed with the STATVIEW software package (SAS). Differences with probability values below 0.05 were considered significant.

**Results**

**Characteristics and Adhesion Molecule Profile of EPCs Derived From Cord Blood**

EPCs isolated from human umbilical cord blood expressed high levels of CXCR4, the sole SDF-1 receptor, and also high constitutive levels of SDF-1 (supplemental Figure 1A and IB). They expressed significantly more CXCR4 and SDF-1 mRNA than did mature endothelial cells (HUVECs). CXCR4 receptor endocytosis occurred rapidly at all SDF-1 concentrations (supplemental Figure IC). In our experimental conditions, freshly isolated EPCs responded to SDF-1. SDF-1
enhanced their proliferation, migration, and differentiation into capillary-like structures on Matrigel and their extravasation through HUVEC monolayers (supplemental Figure II).

Like HUVECs, freshly isolated EPCs expressed α2, α4, α5, α6, α5, and β1 integrin chains but little or no β2 and β4 (supplemental Figure IIIA). Subunit α2 and α6 integrin chain membrane expression levels, determined by quantitative flow cytometry, were significantly higher on EPCs than on HUVECs (5-fold and 14-fold higher, respectively; supplemental Figure IIIA and IIIB). The basal gene expression of these integrin chains was compared in EPCs and HUVECs by means of quantitative RT-PCR (supplemental Figure IIIB). Transcripts of α5, α6, and α5 were respectively 2-, 2-, and 4-fold more abundant in EPCs than in HUVECs. Furthermore, when EPCs were exposed for 15 min to a laminar shear stress of 50 s⁻¹ (venous shear conditions), fluorescence-activated-cell sorter (FACS) analysis showed an increase in the expression of αM (50%, P<0.001) and β2 (35%, P<0.01) compared with control floating EPCs (supplemental Figure IIIIC). These cell surface integrins are involved in cell adhesion to the endothelium. These results suggest that EPCs display stronger homing capacity than mature endothelial cells.

We then examined the effect of SDF-1 stimulation on integrin and proangiogenic growth factor expression levels and gene expression. EPC exposure to SDF-1 for 30 min resulted in upregulation of cell-surface α4 (6%, P<0.01) and αM (67%, P<0.001) integrin subunit expression (data not shown). Real-time RT-PCR was also used to determine mRNA levels of the proangiogenic factors VEGF-A and FGF2. No change was observed in VEGF-A or FGF-2 mRNA levels. In the same experimental conditions, FGF-2 release increased 1.5-fold (P<0.05) in media conditioned by SDF-1-stimulated EPCs, whereas no significant increase of VEGF release was observed as measured with an ELISA kit.

To examine whether preconditioning of EPCs with SDF-1 could further promote their recruitment in vitro, EPCs were first incubated in starvation medium overnight, then stimulated with SDF-1 (100 ng/mL) for 1 min to 48 h, washed, and used in angiogenesis and adhesion assays.

**Figure 1.** SDF-1–stimulated EPCs showed enhanced adhesion to activated endothelium at 50 s⁻¹. A, Adherence of treated EPCs to activated HUVECs. B, Fraction of adherent and tethering cells in each experimental group. C, Resistance to detachment by increased shear stresses from 50 to 2500 s⁻¹. D, Involvement of ICAM and VCAM. Control (CTRL, □), SDF-1–pretreated-EPCs (SDF-1, △), and AMD3100 then SDF-1–pretreated-EPCs (AMD3100, ●). *P<0.05 ***P≤0.001.

**SDF-1–Stimulated EPCs Show Enhanced Adhesion to Stimulated HUVECs in Physiological Shear Stress**

This dynamic model was used to simulate the shear forces encountered by EPCs when they adhere to vascular endothelial cells. No EPCs adhered to unstimulated endothelium. EPC adhesion was dependent on endothelial activation and on the shear rate, as it was optimal at shear rates below 350 s⁻¹, whereas far fewer cells adhered at 500 s⁻¹. SDF-1–stimulated EPCs adhered during the first few minutes (much more rapidly than control EPCs). As shown in Figure 1A, short EPC exposure to SDF-1 caused a rapid increase in adhesion to HUVECs (55±20 treated cells/cm² versus 15±12 control cells/cm²; P<0.001). This effect was of the same order with EPCs treated for 30 min and 24 h. Adhesion of SDF-1–stimulated EPCs to flow-activated HUVECs was strongly reduced (P<0.001) when cells were pretreated with AMD3100, a specific antagonist of CXCR4 (Figure 1A). The observed adhesion process was thus dependent on CXCR4 activation. SDF-1 pretreatment increased the rate of cell tethering to the activated endothelium by about 40% at 50 s⁻¹ (Figure 1B). SDF-1–stimulated EPCs adhered firmly to the endothelium and were more resistant than control cells to washing at shear rates up to 2500 s⁻¹ (Figure 1C).

Treatment with AMD3100 before SDF-1 stimulation increased the percentage of adherent EPCs (8% versus 5% CTRL, P<0.05; Figure 1B). However, these SDF-1–pretreated and AMD-EPCs showed poor resistance to detachment (Figure 1C). It is known that SDF-1 interacts with cell surface heparan sulfate proteoglycans (HSPGs) with relative high affinity in addition to its receptor CXCR4. Our data suggest that SDF-1–stimulated AMD-EPC activation triggered by SDF-1 bound to HSPGs is not sufficient to induce firm adhesion.

Adhesion was further reduced, by 40 and 60%, respectively, by HUVEC treatment with functional blocking antibodies against ICAM-1 or VCAM-1 (P<0.001), suggesting a potential role for α5β2 (LFA-1), α5β2 (MAC-1), and α4β1 (VLA4), their respective ligands, in the recruitment of SDF-1–stimulated EPCs to HUVEC monolayers (Figure 1D).

**SDF-1–Stimulated EPCs Show Enhanced Differentiation Into Vascular Tubes in Matrigel**

Capillary-like tube formation on Matrigel was also enhanced when EPCs were stimulated with SDF-1. EPCs displayed increased formation of tubular structures when examined microscopically after being plated on Matrigel after 1 min of SDF-1 exposure (Figure 2A and 2B). No tubular structures were observed with untreated EPCs. Treatment for 30 min, 24 h, and 48 h led to a significantly more extensive tubular network (1.6-fold, P<0.001). Immunofluorescence studies showed that SDF-1 pretreatment caused cytoskeleton rearrangement with stress fiber formation; this started 1 to 2 min after stimulation, was maximal at 30 min, persisted at 24 h, and disappeared after 48 h (Figure 2C). The tube formation...
and the reorganization of filamentous actin induced by SDF-1 was completely abrogated by AMD3100. Gelatin zymography of medium conditioned by SDF-1–stimulated EPCs revealed that tube formation coincided with increased secretion of pro–MMP-2, within minutes (1.5-fold, \( P<0.05 \) Figure 3A and 3B). SDF-1 stimulation thus triggers EPC adhesion to activated endothelium and induces vascular tube formation through cytoskeleton reorganization and FGF-2 and pro–MMP-2 secretion.

**SDF-1 Prestimulation Enhanced EPC Therapeutic Potential**

By transplanting human EPCs into nude mice, we confirmed the positive effect of SDF-1 prestimulation. Intravenous injection of SDF-1–stimulated EPCs raised the ischemic/nonischemic limb angiography score by 60% compared to control animals injected with untreated EPCs (\( P<0.01 \)) (Figure 4A). Capillary density was 65% and 30% higher in mice transplanted with pretreated cells compared with PBS-treated and control EPC-injected mice, respectively (\( P<0.01 \);

![Figure 2](http://example.com/figure2.png)

**Figure 2.** SDF-1–stimulated EPCs showed an enhanced capacity to differentiate into vascular tubules in Matrigel. A, Quantification of tubular structures in Matrigel. B, Light micrographs showing typical tubules. C, Actin polymerization observed by confocal microscopy (40× objective). Results are the mean±SEM of 3 determinations ***\( P<0.001 \), **\( P<0.01 \) n=3. CTRL indicates control untreated EPCs.

![Figure 3](http://example.com/figure3.png)

**Figure 3.** SDF-1–stimulated EPCs showed enhanced secretion of pro–MMP-2. A, Gelatinolytic activities of metalloproteinases detected in culture supernatants by zymography. B, Quantitative analysis of gelatinolytic activities. MMP-9 □, MMP-2 ■. Results are the mean±SEM of 3 determinations, *\( P<0.05 \) n=3. CTRL indicates control untreated EPCs.

![Figure 4](http://example.com/figure4.png)

**Figure 4.** SDF-1–stimulated EPCs showed enhanced proangiogenic potential in hindlimb ischemia. Quantitative analysis of microangiography (A), capillary density (B), and foot perfusion (C) in mice injected with PBS, untreated EPCs (CTRL), and SDF-1–stimulated-EPCs (SDF-1). Values are expressed as means±SEM; n=10 per group. *\( P<0.01 \), **\( P<0.001 \) vs PBS-injected mice; \#\( P<0.05 \) ##\#\( P<0.001 \) vs control EPC-injected mice.

Figure 4B). Intravenous delivery of SDF-1–stimulated EPCs resulted in 25% and 60% greater hindlimb blood flow recovery than in control EPC-treated (\( P<0.05 \)) and PBS-injected (\( P<0.001 \)) animals, respectively (Figure 4C). These results demonstrate that SDF-1 prestimulation strongly enhances the therapeutic potential of EPCs in nude mice with hindlimb ischemia.

**Role of CXCR4 and HSPG in Vascular Tube Formation and Adhesion of SDF-1–Stimulated EPCs to HUVEC Monolayers**

A 30-minute SDF-1 exposure period was chosen to assess the effects of SDF-1 pretreatment on EPC functional activities in greater depth. Because HSPGs play a key role in chemokine presentation to circulating cells,23 we investigated the role of EPC surface HSPGs in these phenomena. EPCs were treated for 120 min with enzymes that selectively degrade either heparan sulfate or chondroitin sulfate, and were then incubated with 100 ng/mL SDF-1 at 37°C for 30 min. EPC treatment with heparinase III (which specifically removes heparan sulfate) before SDF-1 pretreatment drastically re-
part of the chamber (Figure 6B3). This lag time in EPC adhesion might correspond to the period of SDF-1 uptake.

Discussion

SDF-1 and its transmembrane receptor CXCR4 play a pivotal role in vasculogenesis and regulate the trafficking of immature stem cells. Enhanced SDF-1 expression and production in the endothelium is essential for the engraftment of human stem cells and for their maintenance in situ. Ex vivo exposure of EPCs to SDF-1 before their injection would be expected to enhance their recruitment from the circulation to ischemic tissue and, thus, their graft efficiency.

We examined whether SDF-1 pretreatment led to firm EPC adhesion to a HUVEC model of vascular endothelium in physiological shear flow conditions, as well as their differentiation into capillary tube structures. We first show that EPCs adhere preferentially to activated HUVEC monolayers in low-flow conditions comparable to those prevailing in the vasculature where angiogenesis takes place. No EPC adhesion to nonactivated HUVECs was observed. In vivo, EPCs generally home to areas of altered shear stress, where the endothelium is activated. These areas are believed to have an effect on the adhesion and extravasation of progenitor cells to ischemic sites, promoting firm adherence to activated endothelium.

We then found that SDF-1 priming leads to a concentration- and time-dependent increase in EPC angiogenic activities. SDF-1 pretreatment enhanced shear-resistant EPC adhesion to activated HUVEC monolayers. SDF-1 pretreatment also promoted extravasation of treated EPCs (data not shown) and their differentiation into tubular structures. These effects involved CXCR4/SDF-1 interaction, as they were significantly reduced after EPC preincubation with AMD3100. As a consequence, SDF-1–stimulated EPCs were more potent than untreated cell for therapeutic revascularization in a model of hindlimb ischemia.

SDF-1 stimulation also upregulated the expression of cell-surface α4 and αM integrin subunits involved in cell homing to the neovasculature and the secretion of FGF-2 and pro-MMP-2, which are involved in enhancing cell invasion and vascular remodeling. Our data are in agreement with those of Kodali et al, who underlined the role of SDF-1 in the regulation of pro-MMP-2 levels. The cell surface integrin α4 and αM, combined with respectively β1 and β2, form α4β1 (VLA4) and αMβ2 (MAC-1) involved in cell adhesion to the endothelium. In flow conditions, anti-VCAM and anti-ICAM antibodies were both highly effective in blocking SDF-1–pretreated EPC adhesion to activated HUVECs, suggesting that firm adhesion of SDF-1–stimulated EPCs is related to ICAM-1 and VCAM-1 expression on the endothelium. Activation of integrins α4β1 and αMβ2 are thus crucial for regulating the adhesion of SDF-1–stimulated EPCs to HUVEC monolayers. However, CXCR4 activation by its ligand SDF-1 was not sufficient: AMD3100 treatment before SDF-1 exposure led to an increase in cell adhesion, but the cells exhibited poor resistance to detachment. These findings may help to understand why AMD3100 diminishes CXCR4+ cell incorporation in vivo. In addition, abrasion of HSPGs on pretreated EPCs led to a similar decrease in cell adhesion and differentiation, as EPCs treated with...
heparinase or chondroitinase did not differentiate, migrate, or adhere to the endothelium. HSPGs are not only involved in stress fiber formation and the size of focal sites of adhesion in HUVECs\textsuperscript{23} but also bind and present SDF-1 to endothelial cells.\textsuperscript{22}

To summarize SDF-1 anchored to HSPGs of pretreated EPCs permits rapid EPC activation via its interaction with its receptor CXCR4. This triggers EPC adhesion by upregulating $\alpha_4\beta_1$ and $\alpha_6\beta_1$ integrin expression. The weak expression of selectins by EPCs (data not shown) does not affect the arrest of these cells in flow conditions; several reports show that VLA4 can mediate both tethering and firm adhesion.\textsuperscript{29} SDF-1 pretreatment triggered $\alpha_4\beta_1$ and $\alpha_6\beta_1$ on the EPC surface to bind VCAM-1 and ICAM, resulting in firm EPC adhesion to activated endothelium. In addition to this established role of SDF-1 in integrin activation, our data suggest a SDF-1 transportation from EPCs-HSPGs to HUVECs-HSPGs which has a positive effect on these events. On arrest on HUVECs, SDF-1 facilitates EPC extravasation through HUVECs. SDF-1–EPC preactivation enhances EPC migration and differentiation through the secretion of pro–MMP-2 and the proangiogenic growth factor FGF-2. HSPGs and immobilized SDF-1 synergistically stimulate EPCs to roll along and adhere to the endothelium.

Ex vivo EPC priming with SDF-1 before transplantation may thus provide an effective therapeutic strategy to improve the recruitment of injected cells to sites of neovascularization and thereby enhance the efficiency of cell therapy for ischemic vascular diseases.

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**Disclosures**

None.

**References**


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Ex vivo priming of EPC with SDF-1 before transplantation could increase their vasculogenic potential

ZEMANI: Preconditioning of EPC with SDF-1 and proangiogenic activity

Faouzia Zemani, Jean-Sébastien Silvestre, Françoise Fauvel-Lafeve, Arlette Bruel, José Vilar, Ivan Bieche, Ingrid Laurendeau, Isabelle Galy-Fauroux, Anne Marie Fischer and Catherine Boisson-Vidal

Expanded methods for online publication

Cell culture

Umbilical cord blood was diluted in an equal volume of HBSS, and mononuclear cells were isolated by density-gradient centrifugation with 1.077 g/ml Histopaque solution (Sigma Chemicals) as described by Bompais et al [Bompais H et al Blood 2004; 103: 2577-84]. Plastic-non adherent cells were enriched in CD34+ cells (90% purity) by using immunomagnetic beads and the MACS technique (Miltenyi Biotec). Cells were then plated on 0.2% gelatine-coated 24-well plastic culture dishes at a density of 5x10^5/ml and maintained in endothelial basal medium (EBM-2; BioWhittaker, Cambrex) supplemented with SingleQuots endothelial growth medium and 5% FCS as previously described [Smadja DM et al Arterioscler Thromb Vasc Biol 2005; 11:2321-7]. When an EPC colony became visible microscopically, the cells were detached with trypsin-EDTA (Eurobio) and expanded for further analysis.

Endothelial cells from human umbilical cords (HUVEC) were isolated by enzymatic digestion as previously described [Matou S et al. Thromb.Res. 2002; 106: 213-21]. EPC and HUVEC
were characterized as previously described [Smadja D et al. ATVB. 2005; 25: 2321-2327]. FACS was used to detect the expression of cell-surface antigens of the endothelial lineage.

**CXCR4 receptor endocytosis assay**

Receptor downregulation was performed as previously described [Burger M. et al. Oncogene. 2003; 22: 8093-8101]. Briefly, EPC were adjusted to a concentration of 5x10^6 cells/ml in EBM2, 5% SVF. The cells were incubated with SDF-1 at increasing concentrations for 1h at 37°C in 5% CO₂. EPC were washed with a 20-fold volume of ice-cold buffer without FCS, stained with saturating concentrations of PE-labeled antiCXCR4 mAbs (clone 12G5, R&D systems) for 30 min on ice, washed twice with ice-cold buffer and then analysed by flow cytometry.

**Flow cytometry**

Expression of cell-surface antigens on SDF-1-treated and untreated EPC was analysed by measuring immunofluorescence with a FACS-SORT flow cytometer (BD Biosciences). Labelling with mouse anti-human α5 (CD49, clone VC5, BD Biosciences), αL (CD11a, clone 38, Abcys), αM (CD11b, clone 44, Abcys), β2 (CD18, clone MEM 48, Abcys) and ICAM-1 (CD54, clone 15.2, Abcys) was visualised by using RPE-conjugated goat anti-mouse F(ab’)₂ (Beckman Coulter). Anti-human α4 (CD49d, clone 9F10, BD Biosciences), α6 (CD49f, clone NKI-GoH3, Abcys), β4 (CD104, Pharmingen International), VCAM (CD106, clone P3C4, Abcys) and SDF-1 receptor (CXCR4 clone 12G5, R&D systems) were used directly PE-conjugated. Mouse monoclonal antibodies against α2 (CD49b, clone AK-7, BD Biosciences) and β1 (CD29, clone 353, Valbiotech) were used directly FITC-conjugated. In each immunofluorescence experiment isotype-matched mouse IgG1 or IgG2 antibodies (from the same manufacturer as the immune antibodies) were used as a negative control and the
fluorescence intensity of stained cells was gated according to established methods. Data were analysed with CellQuest™ software (Becton Dickinson).

**Immunofluorescence staining and microscopic analysis**

EPC seeded on multiwell slides were fixed with 4% paraformaldehyde and incubated for 30 min at 4°C with monoclonal anti-human CXCR4 (clone 12G5, R&D Systems) in PBS-1% FBS and then labelled for 30 min at 4°C with a Alexa Fluor 488 complex (Molecular Proje). For internalization experiments, cells were incubated for 30 min at 37°C prior to fixation. After fixation, cells were permeabilized with 0.1% Triton-X-100 in PBS and non specific binding sites were saturated with PBS-10% FBS for 30 minutes. As controls, cells were incubated with the isotypes. Actin was visualized by using phalloidin coupled to FITC (2 mM, Sigma - Beckman Coulter). The cells were coverslipped with mounting medium and Dapi (Vectashield, Biovalley) was used to identify cell nuclei by fluorescence microscopy.

**Real-time quantitative RT-PCR**

The theoretical and practical aspects of real-time quantitative RT-PCR on the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems) have been described in detail elsewhere [Bieche I et al. Cancer Res 2001;61:1652-8]. Briefly, total RNA extracted from SDF-1-stimulated and unstimulated EPCs was reverse-transcribed before real-time PCR amplification. Quantitative values are obtained from the threshold cycle (Ct) number at which the increase in the signal associated with exponential growth of PCR products begins to be detected using PE Biosystems analysis software, according to the manufacturer's manuals. The results, expressed as N-fold differences in target gene expression relative to an endogenous RNA control (the TBP gene, coding for the TATA box-binding protein), termed "Ntarget", are determined as follows: Ntarget = 2^ΔCt_{sample}, where the ΔCt value of the sample is determined by subtracting the average Ct value of the target gene from the average
Ct value of the TBP gene. The N_{target} values of the samples were subsequently normalized such that the HUVEC or unstimulated EPC sample N_{target} value was 1. The nucleotide sequences of primers for TBP and the 10 target genes are shown in Table I. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons. PCR was performed with the SYBR® Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems), in duplicate for each data point. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 65°C for 1 min.
### Supplementary Table:

**Table I:** Oligonucleotide primer sequences used for QRT-PCR analysis of the major integrin subunits ($\alpha_1$, $\alpha_4$, $\alpha_5$, $\alpha_M$, $\beta_1$ and $\beta_4$), VEGF-A, FGF2 and a control gene (TBP).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence</th>
<th>PCR product size (bp)</th>
</tr>
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</table>
| $\alpha_2$ (ITGA2) | U1 : 5’ – AGG TGC CG CAG AAG AAT ATG GT – 3’  
L1 : 5’ – GAC AAC ATC AGA GGG CTC CTG TAT - 3’ | 87 |
| $\alpha_4$ (ITGA4) | U2 : 5’ – AAC ATG AGC AGA TTG GTA AGG CAT A – 3’  
L2 : 5’- CGA TCC AAG CTT TTT ACC TTT CAT T – 3’ | 65 |
| $\alpha_5$ (ITGA5) | U1 : 5’ – ACC CTG CCG CTC AGA TTT CA – 3’  
L1 : 5’ – ACC TAA AAC CAC ACG GCC AGT CT – 3’ | 97 |
| $\alpha_M$ (ITGAM) | 5’ – GCA TCC GCA AAG TGG TAC GA – 3’  
5’ – TTT TCT CCA TCC GTG ATG ACA ACT – 3’ | 94 |
| $\beta_1$ (ITGB1) | U2 : 5’ – CTT CGG ACT TCA GAA TTG GAT TTG – 3’  
L2 : 5’ – GGT GGT GCA GTT CTG TTC ACT TG – 3’ | 116 |
| $\beta_4$ (ITGB4) | U1 : 5’ – AGA TCA ACT ACT CGG CGA TCC A – 3’  
L1 : 5’ – ACC TTG AAG TTG CAT TCC TCA CA – 3’ | 121 |
| SDF-1 | U1 : 5’ – GCC CGT CAG CCT GAG CTA CA – 3’  
L1 : 5’ – TTC TTC AGC CGG GCT ACA ATC T – 3’ | 129 |
| CXCR4 | U1 : 5’ – ACC ATG GAG GGG ATC ATG ATA TAC A – 3’  
L1 : 5’ – ACA GGG TTC CTT CAT GGA GTC A – 3’ | 87 |
| VEGF-A | U1 : 5’ – CTT GCC TTG CTG CTC TAC CTC C – 3’  
L1 : 5’ – CAT CCA TGA ACT TCA CCA CTG TGT – 3’ | 103 |
| FGF2 | U1 : 5’ – AGA AGA GCG ACC CTC ACA TCA A – 3’  
L1 : 5’ – GGT AAC GGT TAG CAC ACA CTC CTT – 3’ | 87 |
| TBP | U : 5’ – TGC ACA GGA GCC AAG AGT GAA – 3’  
L : 5’ – CAC ATC ACA GCT CCC CAC CA– 3’ | 132 |
**Legends for supplementary figures**

**Figure I**: Freshly isolated human EPC express CXCR4. (A) FACS analysis and in vitro immunolocalization of CXCR4 (40xobjective). (1) non-permeabilized-EPC and (2) permeabilized-EPC immunostained with anti-CXCR4 mAb, counterstained with DAPI. (3) isotype control for CXCR4. (B) mRNA expression profile of SDF-1 and CXCR4 in EPC. Data are expressed as a percentage of mRNA expression in HUVEC (mRNA-fold-ratio). (C) CXCR4 receptor endocytosis in EPC (% of control-EPC). Results are the mean ± SE of 3 determinations ***:p≤0.001, CTRL = untreated-EPC.

**Figure II**: SDF-1 induces a proangiogenic phenotype in EPCs. (A) SDF-1 enhances EPC proliferation after 48 h of incubation at 37°C, in a concentration-dependent manner starting at 20 ng/ml. SDF-1-induced proliferation was similar to VEGF-induced proliferation (40 ng/ml, positive control). (B) SDF-1 induced a concentration-dependent chemotactic response. Incubation with blocking anti-CXCR4 mAbs significantly inhibited EPC migration toward SDF-1 after 6 h of incubation. (C) SDF-1 promotes the formation of capillary-like structures by EPC in Matrigel. (D) SDF-1 mediates EPC extravasation through HUVEC monolayers after 18 h of incubation. * p<0.05; ** p<0.01. CTRL = control, MCP-1 monocyte chemoattractant protein 1, a potent mononuclear cell chemoattractant produced by endothelial cells (negative control).

**Figure III**: Freshly isolated human EPC derived from cord blood express adhesion molecule receptors. (A) FACS analysis of human EPC and HUVEC major integrin subunit α2, α4, α5, α6, αM, αL, β1, β2 and β4 expression profile (n=3 each). The solid histogram indicates the negative staining with the isotype control antibody. (B) Quantification of protein and RNA expression of major integrin subunits (n=3 each) by human EPC. Data are expressed as a percentage of the mean fluorescence intensity of HUVEC (protein fold-ratio) or mRNA...
expression in HUVEC (mRNA fold-ratio). (C): FACS analysis of human EPC showing the
effect of laminar shear stress on the expression of $\alpha_m$ and $\beta_2$. Solid line = EPC control, bold
line = EPC exposed for 15 min to shear stress of 50 s$^{-1}$. Data in A and C are from
representative experiments. Data in B are the average of 3 experiments plus or minus SD. ND
= not determined.
<table>
<thead>
<tr>
<th>mRNA fold-ratio</th>
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<tbody>
<tr>
<td>SDF-1</td>
<td>2.9 +/- 0.6</td>
</tr>
<tr>
<td>CXCR4</td>
<td>4.3 +/- 2.0</td>
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</tbody>
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A

B

C

CTRL 100 500 1000 + SDF-1 (ng/ml)

Mean CXCR4 expression (% of CTRL)

*** *** ***
A

B

<table>
<thead>
<tr>
<th>Protein</th>
<th>mRNA fold-ratio</th>
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<tbody>
<tr>
<td>α2</td>
<td>5.6 ± 3.2</td>
</tr>
<tr>
<td>α4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>α5</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>α6</td>
<td>5.3 ± 2.9</td>
</tr>
<tr>
<td>αM</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>αL</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>β1</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>β2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>β4</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

C